PROPERTIES AND CHANGES OF ALKALINE PHOSPHATASE ACTIVITY DURING AMPHIBIAN DEVELOPMENT

by

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1. INTRODUCTION

In line with the increasing interest in the problem of phosphate compounds in biological processes, the present investigation was undertaken to determine the activity of one of the enzymes capable of hydrolyzing phosphate esters. Alkaline phosphatase was investigated in relation to a special process, namely, embryonic development. Although alkaline phosphatase has been reported previously in embryos (in chick embryos, MOOG (19), and in amphibian embryos, BRACHET (5), KRUGELIS (11)), the present investigation attempts to emphasize the quantitative aspects of the enzyme activity.

The investigation was undertaken to study some of the properties of alkaline phosphatase and to determine the changes of enzyme activity during development. The enzyme activity was determined as the ability of water extracts to liberate inorganic phosphate from sodium glycerophosphate under defined conditions. This was accomplished by a micro-phosphatase method adapted for fractions of single eggs and embryos. The inorganic phosphate liberated was determined by a colorimetric micro-method based on the reduction of phosphomolybdic acid by stannous chloride. The method was modified to measure phosphorus in the range of 4×10^{-3} to 4×10^{-2} μg P.

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2. MATERIALS AND METHODS

2.1. Obtaining of fertilized eggs and care of animals.

The biological materials used for alkaline phosphatase activity were various developmental stages of eggs and embryos of the South African clawed toad, *Xenopus laevis* and the albino Axolotl, *Amblystoma mexicanum*[1].

Fertilized eggs of Xenopus are available at all times of the year by injecting healthy animals with chorionic gonadotropin extract[2]. 50 to 100 I.U. chorionic extract were injected subcutaneously into the female and 10 to 20 I.U. into the male, as suggested by LANDGREBE and SAMSON (13). It was found that the hormonal dosage may have to be doubled during the midwinter months. After injection, the animals are placed in a glass aquarium (25 × 15 × 15 cm) containing 10 cm of non-chlorinated tap water. Coupling occurs within 15 to 24 hours, and up to 2000 fertilized eggs may be collected after 20 to 48 hours.

> The animals were given two months' rest between ovulations in order to insure eggs of good condition. Adult frogs are easily kept in the laboratory. Males and females are kept separate in glass aquaria containing a depth of 20 to 25 cm water which is changed after feeding. The animals are fed pieces of calf liver two or three times a week.

Fertilized eggs of *Amblystoma mexicanum* are available from January to June without hormonal stimulation. A male and female were placed together in 15 cm of non-chlorinated tap water within a tank (40 × 75 × 20 cm) whose inside surface had been painted black. The male sheds spermatophores within 10 to 20 hours, and the female lays 200 to 600 eggs within 20 to 40 hours.

> When not breeding, the salamanders are maintained in the laboratory, being kept singly in glass aquaria and fed calf liver two or three times a week.

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[1] The author gratefully acknowledges the kindness of Dr. H. O. SCHMIT-JENSEN of the Danish State Veterinary Serum Laboratory in supplying the breeding animals for these experiments.

[2] The author thanks the Løvens Kemiske Fabrik of Copenhagen for their generous supply of Chorion-Gonadotropin (Physex Leo 250 i.E.)

2.2. Care and dissection of eggs.

The fertilized eggs of both Xenopus and Axolotl were distributed in batches of 30 to 40 eggs in 150 ml of tap water, which was changed daily. The eggs of Axolotl were allowed to develop at 22.0 °C, eggs of Xenopus at 20.0 °C. Eggs and embryos were designated by the age in hours from the time of egg laying and by the more obvious morphological landmarks of embryonic development.

Dissection of Axolotl eggs[3] for study of parts of gastrulae was made in HOLFRETER's solution without bicarbonate. The vitelline membrane was first removed completely from the gastrulae. By means of glass needles two cuts were made perpendicular to and on either side of the early dorsal lip, leaving a circular strip around the animal-vegetal circumference of the egg. A cut was made at the dorsal lip and the strip laid open. This then was cut into several segments representing various regions along the animal-vegetal meridian which passes through the dorsal lip of the gastrula.

2.3. Preparation of enzyme extracts.

One egg or part of an egg was transferred into a plastic microhomogenizer, whose design has been described by HOLTER and LØVTRUP (10). The egg was ground into a thick brei by rotating the close fitting pestle for several minutes. 50 µl of glass-distilled water were added to the homogenate of Xenopus eggs, and 75 µl to the homogenate of Axolotl eggs. After addition of the water, the brei was mixed by rotating the pestle in the homogenizer for several turns, and the mixture weighed in the homogenizer. The weight (in mg) of the homogenizer and homogenate minus the weight of the empty homogenizer was considered to be equal to the volume (in µl) of the total egg brei. The brei was transferred from the homogenizer into a glass micro-reaction tube (of 250 µl volume) by means of a pipette. 10 µl of chloroform were added to the brei and the mixture centrifuged lightly (30 seconds at 2500×g) to remove yolk and pigment. The chloroform was found necessary to clear the aqueous extract of some suspended particles, possibly fat, which were found to interfere with subsequent colorimetric readings. Addition of small amounts of chloroform at this step did not decrease the activity of alkaline phosphatase.

[3] The author acknowledges the kind help of Dr. JOHN R. GREGG Of Columbia University who did the egg dissections for the experiment on egg parts.

The supernatant extract was pipetted off and its volume determined by weighing the extract in a reaction tube which was covered by a stopper for reaction tubes (see f. inst. GLICK (8), p. 171). This supernatant was then used directly as the source of enzyme and incubated with the substrate. The supernatant of more active extracts from older embryos was first diluted with a known volume of glass-distilled water.

The activity of the enzyme extracts increased so much with development that great dilutions were necessary in the later stages. In the case of Xenopus, the extracts of hatching tadpoles were diluted about 20 times more than the extracts of uncleaved eggs, and in the case of Axolotl, the extracts of hatching tadpoles were diluted 40 times more. 4.0 μl aliquots represented 6.5 per cent of Axolotl uncleaved egg and 0.16 per cent of the Axolotl hatching embryo.

2.4. Incubation of the enzyme extracts with substrate.

The activity of the aqueous extracts has been determined by incubation with sodium glycerophosphate as substrate. The sodium glycerophosphate was obtained from Eimer and Amend Co., New York, and according to the firm was "essentially" a disodium β-gly-cerophosphate. The substrate solution contained the following components:

Sodium glycerophosphate	10 mg/ml or 1.4 mg P/ml
Sodium barbital	40 mg/ml
Magnesium sulfate	2.5 mg/ml

Equal known volumes (about 5 µl) of the enzyme extract and the substrate were placed together into micro-reaction tubes. The volumes were measured out and transferred by means of the Carlsberg constriction pipette (LEVY (14) and LINDERSTRØM-LANG and HOLTER (15); see GLICK (8), pp. 172-176). The pipettes were equipped with a constant water-pressure regulator to ensure delivery of equal volumes. The reaction tubes were closed by stoppers and immersed in a water bath at 40 °C. The enzymatic reaction was stopped by addition of twice the extract volume of 0.25% uranium sulfate in 5.0% trichloroacetic acid (ZITTLE (21)). This reagent was used so that nucleic acid, which was used as substrate in some comparative experiments (to be reported in a future publication) as well as the egg proteins in the brei, be precipitated more completely than with trichloroacetic acid alone. The enzymatic reaction was stopped by

addition of the uranyl reagent at o time, and at various intervals thereafter. All determinations were made in duplicate or triplicate. The precipitates formed were centrifuged at 10,000×g for 3 minutes, and the tubes stored until the phosphate determinations were made.

2.5. Determination of inorganic phosphate.

The method for the determination of inorganic phosphate is based on the reduction of phosphomolybdic acid by stannous chloride, and the reading of the color intensity of the product by a microscopic photometric technique.

The chemical method is a micro-modification of that of KUTTNER and COHEN (12). Although the author is aware of other micromethods for phosphatase and phosphate analysis (see GLICK (8), pp. 124, 208, 210, 226, 280), the present method offers some advantages of simplicity for the material studied in this investigation. The use of stannous chloride as reductant offers greater sensitivity, although as emphasized by BERENBLUM and CHAIN (2), the final acidity as well as the final concentrations of ammonium molybdate and stannous chloride must be controlled with much accuracy. To insure that the final concentrations are within the limits for maximum color development, it was found most satisfactory and convenient to prepare the reagents in concentrations four times the desired final concentration, and to add the four reactants in equal volumes by means of the same constriction pipette.

The reaction mixture thus contained the following:

4 μl sample to be analyzed
4 μl H_2SO_4 ..... 4.0 N
4 μl Ammonium molybdate 3.0%
4 μl Stannous chloride 0.08%

The reagents were all made with glass-distilled water. The ammonium molybdate was stored in the cold in a paraffined bottle. Stannous chloride was made up just before use by dilution with water from a stock solution (40% $SnCl_2$ in concentrated HCl) which is stored in the cold in a brown glass stoppered bottle. The reactants were introduced into a reaction tube in the order mentioned above and mixed after each addition by gentle vibration which was found sufficient for these small volumes. If larger volumes are used, an

electro-magnetic stirrer ("flea") should be introduced for mixing the reagents. 4 µl volumes of each reagent were used giving a final volume of 16 µl which was sufficient for two separate photometric readings on each color reaction.

The reaction may be adapted to somewhat smaller volumes without otherwise changing the technique. Reactions with 2 µl of sample and 2 µl of each of the three reagents were also made, giving a final volume of 8 µl, which could be measured by the photometric system (employing 5.5 µl cuvettes).

2.52. Photometric procedure.

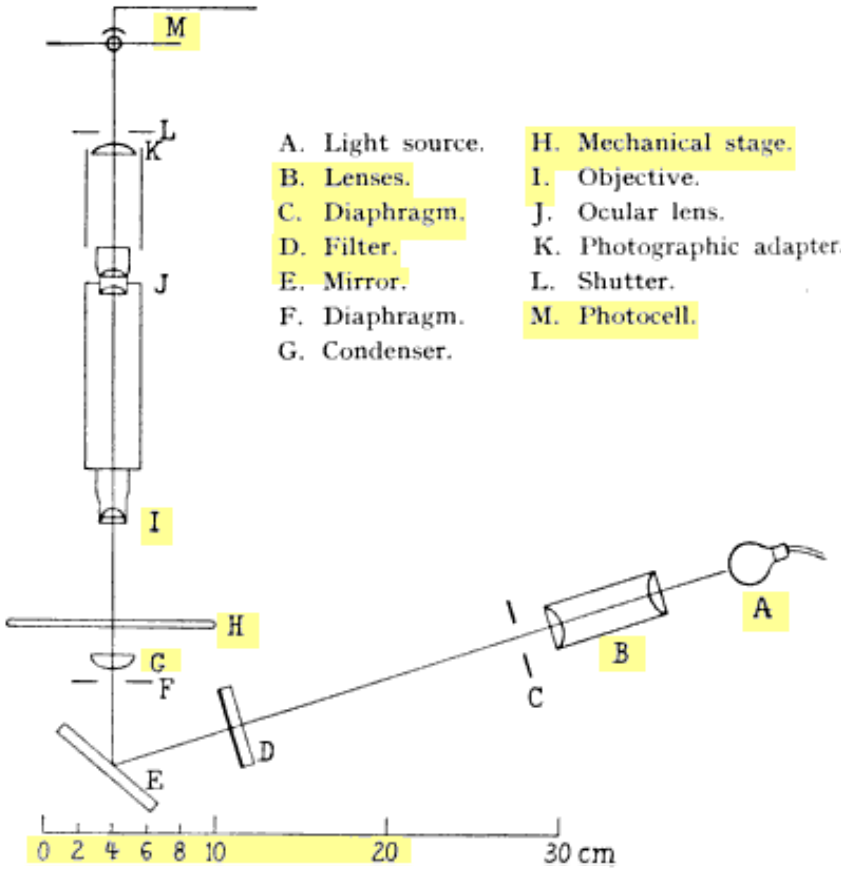

Fig. 1. Diagram of photometric apparatus.

The micro-photometric technique used in this investigation is essentially the same as that described by HOLTER and LØVTRUP (10). The photometric apparatus consisted of a lamp, microscope, photographic adapter, photocell and photometer as shown in fig. I. Except for the light source, the microphotometer employs the same principle

as that described by COMMONER (7). The light source (A) was a Philips Tone film lamp bulb (6 V. 5A. E14) fed by a storage battery. Light-collecting lenses (B) placed in front of the light source acted as secondary source and prevented excess loss of light, and according to the KÖHLER principle used, served as the image. A diaphragm (C) closed down to an opening of 4 mm selected the more parallel rays. A filter (D) of 660 mu was placed in the light path between the lamp and the microscope. The mirror (E) reflected the light to the optical system of the microscope. The iris diaphragm (F) was closed to a 3 mm opening further selecting the light rays so that they formed an almost parallel beam with a focus 3.5 mm above the level of the mechanical stage (H). The diameter of the beam at its focus was about 0.4 mm. The condenser (G) was a Zeiss num. Ap. 1.2 with top lens removed resulting in a system with a focal distance of 31 mm and a num. Ap. 0.4. The microscope was equipped with a low power objective (2 Leitz Wetzlar 6X) (I), a Huygens ocular lens (J) and a Zeiss photographic adapter "phoku" (K) which gave a linear magnification of 18X at the level of the photocell window (M). The latter was attached to a photometer instrument (Electronic photometer model No. 512, Photovolt Corporation, New York).

The solution to be read was placed in a microcuvette which was a thick walled pyrex capillary tube (1.0 mm bore and 7 mm high), ground parallel on both ends, at right angles to the tube. The cuvette was sealed to a glass slide by applying a thin line of vaseline around the outer bottom rim of the cuvette and pressing the cuvette upon the slide. This method of attachment was suggested by Dr. HOLTER and was adapted because the cuvette can be easily detached, cleaned and dried after each measurement by drawing a twisted strip of lens paper through the capillary bore. The solution was introduced into the cuvette by means of a thinly drawn out pipette and covered by a cover slip. The position of the cuvette on the mechanical stage was so adjusted that the light beam went through the center of the capillary bore and the focus was adjusted to be halfway up the cuvette.

After passing through the solution, the light passes through the lens system of the microscope and forms an enlarged image at the level of the photocell window. By opening the shutter (L) in the photographic adapter, the photocell was exposed to the transmitted light and the transmission intensity was measured by the photometer. Measurements were also made of the transmittance

through the glass slide adjacent to the cuvette to correct for any variation in light source and photometer.

The color intensity was measured after 15 to 30 minutes and the quantity of inorganic phosphate as μg was obtained from a calibration curve which has been constructed from measurements with known amounts of phosphate. The standard curve for solutions containing known quantities of phosphate, as determined by the method of this investigation (light path of 7 mm, filter at 660 mμ) is a straight line with a slope corresponding to a ΔE of 0.27 for 0.010 μg P. ΔE is the extinction of the phosphate-containing solutions minus the extinction of the reagents. To obtain greater accuracy, results of several determinations were averaged. The maximum deviation obtained in three independent determinations of the standard curve was found to be 5% of the mean value, while the average standard deviation in five cases was 3% of the mean.

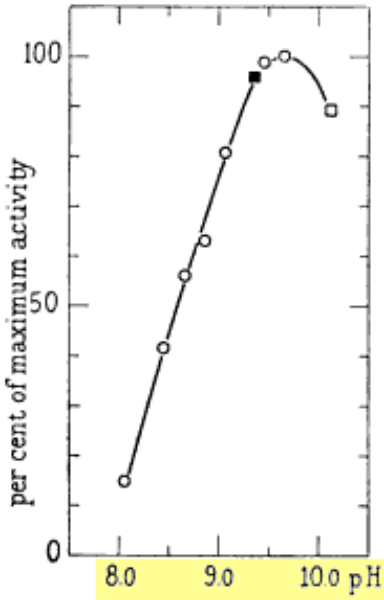

Fig. 2. Correlation between alkaline phosphatase activity and pH. Each point is the average of 3 determinations. ○, □ represent experiments with ammonia buffer, ■ with barbital buffer.

3. PROPERTIES OF ALKALINE PHOSPHATASE

In order to carry out the main part of this investigation, namely the changes of enzyme activity during development, some properties of alkaline phosphatase were first determined.

3.1. Relation between enzyme activity and pH.

Substrate: Sodium glycerophosphate (5.25 mg/ml in final buffer- $MgSO_4$ -enzyme reaction mixture). Buffers: Sodium bartibal 40 mg/ml with 2.5 mg/ml $MgSO_4$ (pH 9.35); NH_2OH (0.1 M) – NH_4Cl (0.1 M) with 2.5 mg/ml $MgSO_4$ (pH 8.0 to 10.0). This latter buffer was used to cover the higher pH range which is not reached with sodium barbital. Incubation: 3 hours at 40 °C. Homogenate: Extract of Axolotl embryo (160 hours old).

The pH of the buffers was checked by means of a glass electrode.

The optimum activity was obtained between pH 9.4 and 9.6. The experiments were carried out with sodium barbital buffer at pH 9.35.

3.2. Relation between enzyme activity and substrate concentration.

Substrate: Varying concentration of sodium glycerophosphate expressed as mg/ml in the total buffer-$MgSO_4$-ensyme reaction mixture. Incubation: 3 hours at 40 °C. Homogenate: Extract of Axolotl embryo (137 hours old).

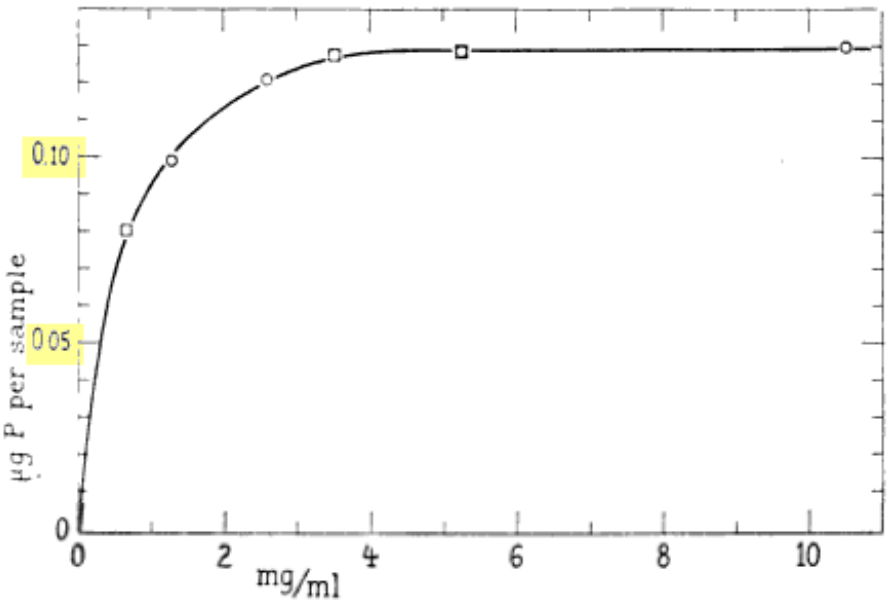

Fig. 3. Correlation between rate of phosphatase activity and substrate concentration.

Abcissa: Substrate mg/ml. Ordinate: µg P liberated.
Each point is the average of 3 determinations. ○, □ represent different experiments with the same extract.

Fig. 3. shows the influence of substrate concentration on the rate of reaction. The substrate concentration of 5.25 mg/ml was chosen for most of the experiments since the rate is essentially independent of substrate concentration at this point.

From the curve, the maximum velocity can be calculated to be approximately 0.13. The half velocity V/2 is 0.065, and corresponds to the substrate concentration of 0.45 mg/ml or 0.0021 M (molecular weight of sodium glycerophosphate being 216.0). Assuming the velocity at 3 hours to be proportional to the initial velocity, the substrate concentration 0.0021 is Ks, the

MICHAELIS Constant or the apparent dissociation constant (LINEWEAVER and BURK (16)). This way of calculation gives only a rough approximation because the above assumption does not actually hold. Yet this value for the MICHAELIS constant is presented since it agrees rather well with that reported in the literature for phospho-monoesterases from other sources, i.e., 0.0025 for kidney phosphatase (AEBI (1)), 0.0019 for bone phosphatase (BODANSKY (3)); 0.0029 for intestinal phosphatase (MARTLAND and ROBINSON (18)).

The rate of reaction of enzyme extracts of Xenopus embryos was also determined with various substrate concentrations. A similar curve of results was obtained for Xenopus extracts, suggesting that the enzymes of the two species have similar characteristics in this respect.

3.3. Relation between enzyme activity and enzyme concentration.

Substrate: Sodium glycerophosphate 5.25 mg/ml in total reaction mixture. Homogenate: Extracts of Axolotl embryo (137 hours old). Incubation: 3 hours at 40° C.

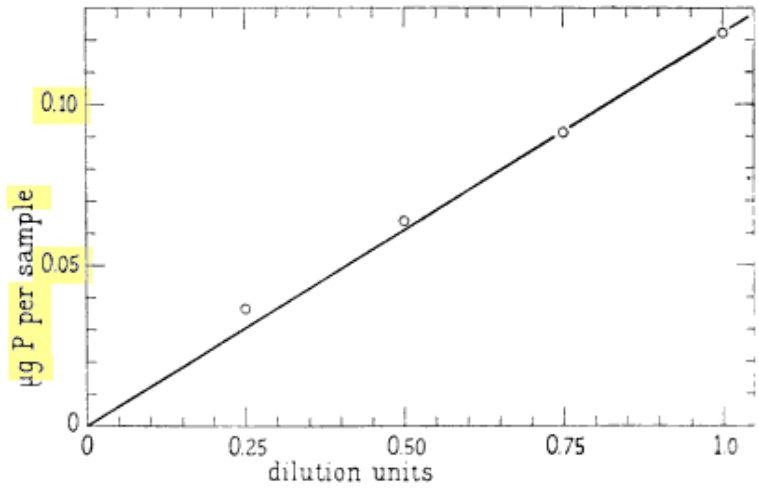

Fig. 4. Correlation between alkaline phosphatase activity and enzyme concentration.

Abcissa: Enzyme dilution units. Ordinate: µg P liberated. Each point is the average of 3 determinations.

Fig. 4 shows a linear relationship between enzyme concentration and enzyme activity under defined conditions. A deviation from the straight line was found when the substrate concentration was one half of the above value, indicating the reaction rate changes

with decreasing amounts of the available substrate. Yet this deviation occurs only when the reaction proceeds to values above 0.15 μg P (equivalent to a ΔE value of 0.9) so that an initial linear relationship exists even with substrate concentrations of one-half maximum velocity value which were used in the Xenopus experiments. ΔE values above 0.9 were discarded.

3.4. Relation between enzyme activity and time.

Substrate: Sodium glycerophosphate 2.25 mg/ml in total reaction mixture. Incubation: 40 ° C. Homogenate: Extracts of Axolotl embryo (137 hours old) and Axolotl uncleaved egg.

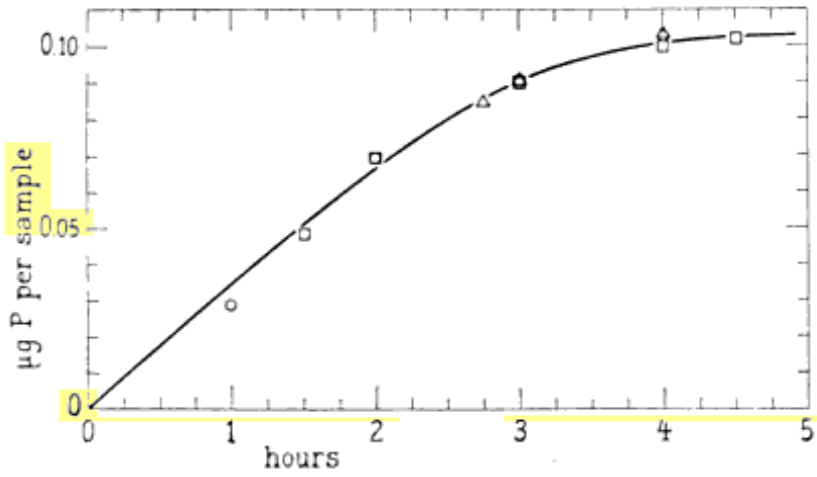

Fig. 5. Correlation between enzyme activity and time.

Ordinate: μg P liberated.

○, □ represent two experiments on the same extract of Axolotl embryo. △ represents experiments on extract of Axolotl uncleaved egg. The point at 3 hours is an arbitrarily chosen coincidence point to allow graphical alignment of the curves. Each point is the average of 3 determinations.

The curve shows that alkaline phosphatase activity is not constant. The rate of reaction decreases with time. Since the substrate concentration was such as to give essentially the maximum velocity, and the enzyme inactivation by heat presumably is negligible (at this reaction time), another reason for the decrease in rate must be sought. There is the possibility that inorganic phosphate liberated may inhibit the reaction and thus change the rate. Fig. 5 shows that the enzyme activity of both uncleaved egg and 137 hour old embryo have the same relationship with time. Although not shown on the curve, similar experiments were performed for other

Axolotl stages (blastula, dorsal lip, and hatching stages) and all curves superimpose upon the one presented when one point at 3 hours is taken as the arbitrary point of coincidence. This suggest that the alkaline phosphatase activity of all embryonic stages of Axolotl is due to the same enzymes of very similar characteristics.

The relation of enzyme activity with time in the case of both Axolotl extracts and Xenopus extracts is very similar, suggesting that the enzyme activity in these forms is due to similar enzymes.

3.5. Relation between enzyme activity and temperature.

Substrate: Sodium glycerophosphate 2.2 mg/ml in total reaction mixture. Incubation: Varying temperatures for 4 hours 50 minutes and for 7 hours. Homogenate: Xenopus embryo extracts.

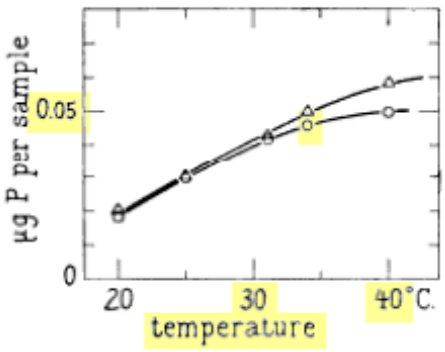

Fig. 6. Correlation between enzyme activity and temperature during two different lengths of incubation time

Ordinate: µg P liberated.

○ represents incubation for 7 hours (neurula extract); △ represents incubation for 4 hours 50 minutes (tadpole extract). Each point is the average of 3 determinations.

A decrease of rate at the higher temperature is shown in fig. 6. During the longer incubation time the greater decrease of rate is most likely due to inactivation of enzyme. Due to the low activity of the extracts of the early stages of Xenopus, the reactions were allowed to proceed for 8 hours at 40 °C. This would probably result in a certain amount of enzyme destruction. Since the enzyme activity is a linear function of the concentration of the enzyme, and all the extracts were diluted to give about the same enzyme activity, the inactivation of the enzyme would be approximately equal in all extracts and a relative comparison of the Xenopus extract activity is valid. For Axolotl extracts, the incubations were carried out for 3 hours at 40 °C. These conditions would not result in any significant of enzyme inactivation as seen from fig. 6.

4. CHANGES IN ENZYME ACTIVITY DURING DEVELOPMENT

4.1. Relation between enzyme activity and developmental stages in Xenopus laevis.

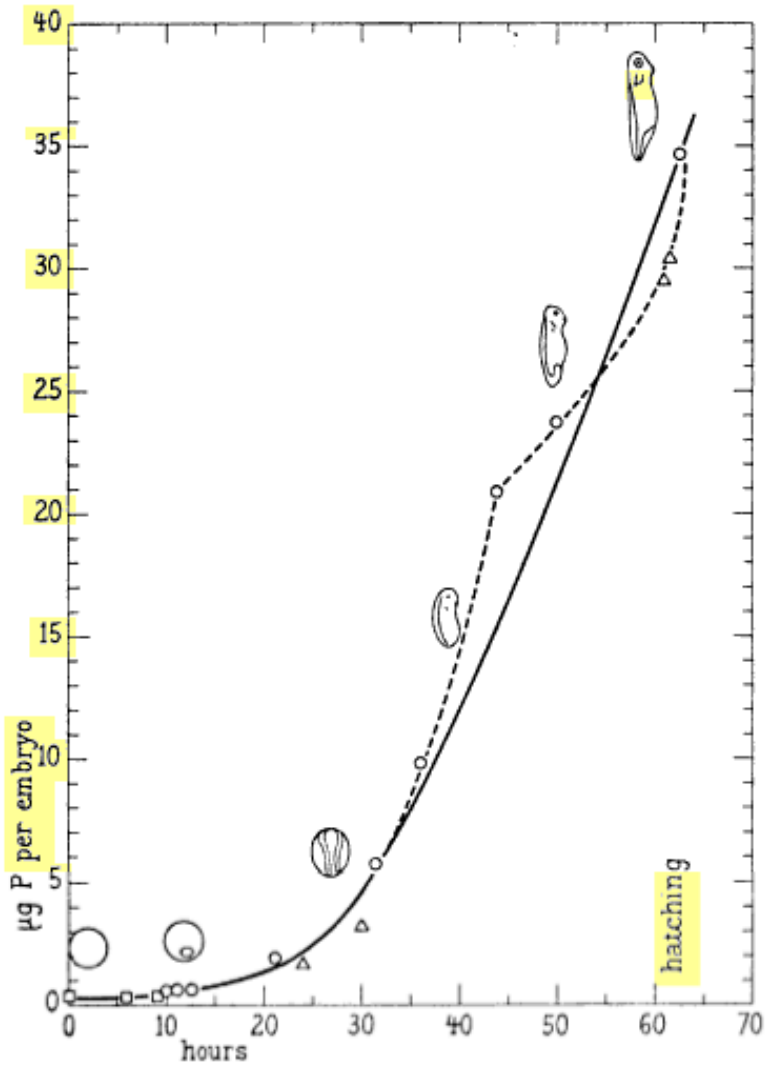

Fig. 7. Correlation between alkaline phosphatase activity and the developmental age of the embryos of *Xenopus laevis*.

Abcissa: Developmental time in hours. Ordinate: µg P liberated per embryo. Substrate: Sodium glycerophosphate 2.2 mg/ml.

○, △ represents eggs laid at 2 different times by the same female. □ represents eggs of a different female. Each point is the average of 2–3 determinations on extracts of the same embryo.

The μg P per embryo was calculated in the following way:

μg = P/embryo = μg P from Δ E value × correction constant for the fraction of sample used for the photometric readings × dilution factor × correction for time variation.

Fig. 7 is presented only for a relative comparison of the developmental stages. There is some destruction of the enzyme at 40 °C. for the longer time of incubation. The inactivation is roughly estimated to be about 5%. Other unknown factors affecting the enzyme activity may be present in the unpurified extract of the embryos.

4.2. Relation between enzyme activity and developmental stages of Amblystoma mexicanum.

The results from figs. 7 and 8 show a steady increase in the alkaline phosphatase activity during embryonic development of both *Xenopus* and *Amblystoma*. The activity curves are essentially alike and probably reflect similar processes in the early development of both species. A correlation of enzyme activity with any known process in the embryo is difficult to make. The increase in activity can not be directly correlated with the mitotic rate since data of BRAGG (6) on *Bufo cognatus* show that the mitotic rate declines greatly at the end of gastrulation, and it is at the end of gastrulation that the enzyme increase begins to be considerable. The increase is more likely to be correlated with organogenesis than with mitosis.

When the enzyme activity increase is plotted logarithmically there is evidence of a "break" in the curves at 140 hours in Axolotl development and 44 hours in Xenopus development. Since the data are based on single embryo determinations, variations in individual size of embryos can not be accounted for, and thus the 'break' may be due merely to embryo size differences.

'Breaks' have been reported from the data of various investigations relating to oxygen consumption (see BOELL (4)). A 'break' appears at about 6th to 7th day of development of several species of *Amblystoma* and after the end of gastrulation in several species of *Rana*. These 'breaks' correspond roughly in developmental time to the 'breaks' in the phosphatase activity increase.

Although the possible presence of a 'break' in the slope of the phosphatase activity curve can not be ignored, the present data furnish no conclusive evidence.

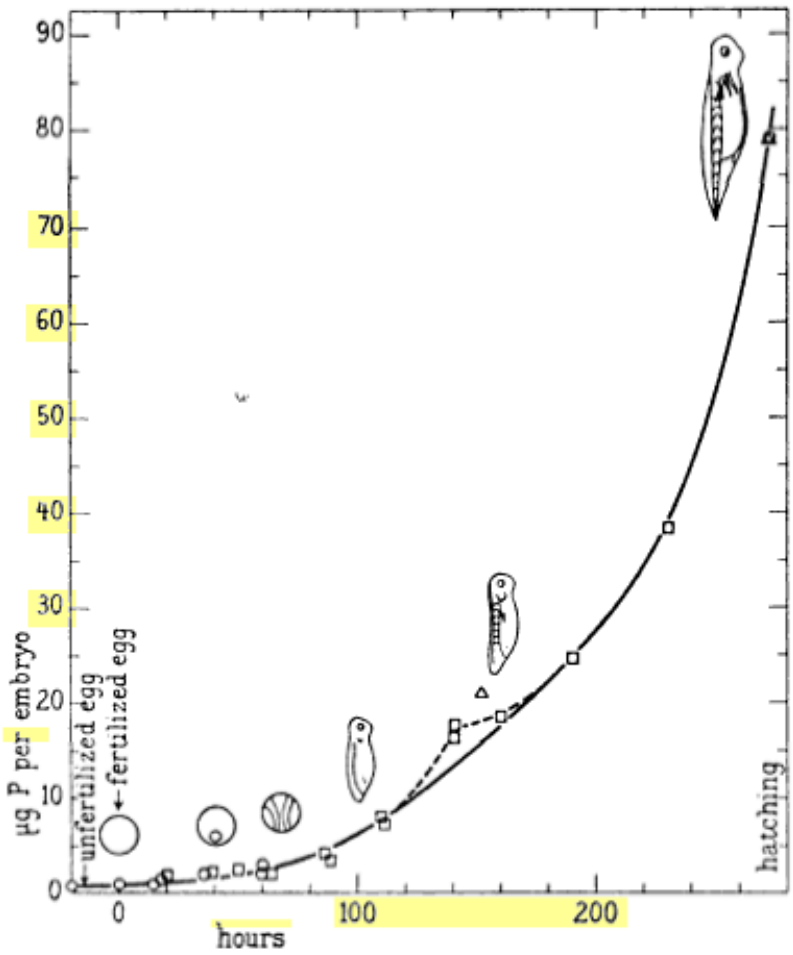

Fig. 8. Correlation between alkaline phosphatase activity and the developmental age of the embryos of *Amblystoma mexicanum*.

Abcissa: Developmental time in hours. Ordinate: µg P liberated per embryo. Substrate: Sodium glycerophosphate 5.25 mg/ml.

○, □ represent eggs laid at two different times by the same female. △ represent eggs laid by a different female. Each point is the average of 2–3 determinations on the extract of the same embryo.

An interesting question arises concerning the distribution of the enzyme in the various regions of the egg. To give some insight into this problem, eggs in the stages of early gastrulation were dissected into segments around the dorsal-ventral (animal-vegetal) circumference. The dissections are described by GREGG and LØVTRUP (9). The reduced weight of the explants was determined by the diver balance method of ZEUTHEN (20) and LØVTRUP (17). Breis

of the explants were made in HOLFRETER's solution and aliquots were used to determine phosphatase activity and total nitrogen content.

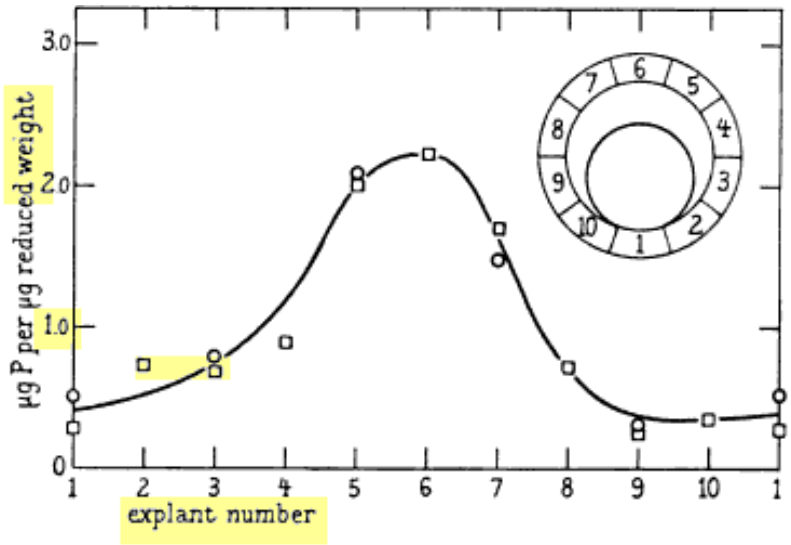

Fig. 9. Correlation of the region of the Axolotl egg to the ratio of phosphatase activity over reduced weight.

Abcissa: Region of egg corresponding to the numbered regions in the regions in the sketch in the upper right hand corner of the graph. Incubation time: 3 hours at 40 °C. Substrate: 5.25 mg/ml sodium glycerophosphate.

The results show a regional difference of alkaline phosphatase activity in the early gastrulae. The regions of the animal pole have a higher enzyme activity than those of the vegetal pole with respect to reduced weight. When the ratio of μg P per μg total N is plotted against the explant number, the same curve as fig. 9 results (see GREGG and LØVTRUP (9)). These data show that the enzyme is not abundant in the yolk platelets which are most numerous in the vegetal regions. The enzyme seems rather a part of the non-yolk components of the cells.

The author is sincerely grateful to Dr. HEINZ HOLTER for his interest, guidance and encouragement during the investigation of this problem as well as for the generous hospitality extended to her at the Carlsberg Laboratory. To Magister SØREN LØVTRUP I also express thanks for helpful discussions and assistance in many aspects of the work.

5. SUMMARY

By means of a new micro-phosphatase method, the alkaline phosphatase activity was determined in water extracts of embryos of *Xenopus laevis* and *Amblystoma mexicanum*. The activity was determined as the ability of the extracts to liberate inorganic phosphate which was measured by a colorimetric method modified to determine 4×10^{-3} to 4×10^{-2} μg P.

Some properties of the enzyme and the changes of enzyme activity during various developmental stages were determined. The activity was found to increase steadily during development of both forms.

Segments around the animal-vegetal circumference of the gastrula showed a greater phosphatase activity in the animal over the vegetal pole with respect to μg reduced weight and total nitrogen.

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